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The Potential Roles of Melanopsin Signaling in Mediating the Effects of Environmental Light on Voluntary Ethanol Intake in Mice

Rachel Brooks

THE POTENTIAL ROLES OF MELANOPSIN SIGNALING IN MEDIATING THE EFFECTS OF ENVIRONMENTAL LIGHT ON VOLUNTARY ETHANOL INTAKE IN MICE

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Rachel Brooks

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Advisory Committee:

Dr. Alan Rosenwasser, Professor of Psychology, Advisor

Matthew Hartman, PhD Candidate in Biomedical Sciences

Dr. Sally Molloy, Professor of Microbiology

Dr. Kristy Townsend, Professor of Neurobiology

Dr. Molly Maclean, Professor of Biology

ABSTRACT

Intrinsically photosensitive retinal ganglion cells (ipRGCs) comprise a small subset of photoreceptors found in the eye containing the newly discovered photopigment, melanopsin. ipRGCs project directly to the hypothalamic suprachiasmatic nucleus (SCN), the central "pacemaker" underlying the generation and entrainment of circadian rhythms. Photic stimuli detected by ipRGCs are transmitted to the SCN via the retinohypothalamic tract (RHT), mediating the entrainment of the SCN pacemaker. In addition to circadian entrainment, these pathways may also contribute to seasonal changes seen in both animals and humans, such as seasonal breeding cycles in animals and seasonal affective disorder in humans. Our lab has recently found that changes in the laboratory lighting environment can alter voluntary alcohol intake in mice, which may be related to the seasonal variation in alcohol use seen in humans. In this study, we examined the possible role of melanopsin signaling in mediating the effects of photoperiod on alcohol intake. Male and female melanopsin knockout (*Opn4* -/-) and wild-type control mice of the same genetic background were housed individually in running-wheel cages and initially kept on a 12:12 light-dark (LD) cycle for 3 weeks, followed by constant light (LL) or constant darkness (DD) for 3 weeks, then returned to LD 12:12 for the final 3 weeks. Animals had continuous access to running wheels, plain water, and 10% ethanol solution throughout the experiment. Wheel turns were monitored by a computer interface and ethanol and water intake were recorded manually at weekly intervals. While $Opn4^{-/-}$ mice showed the expected reductions in circadian light sensitivity from controls, the two genotypes displayed identical reductions in ethanol intake under LL and DD. Thus, melanopsin-based photoreception is not necessary for light-induced changes in alcohol preference drinking in mice.

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INTRODUCTION

1.1 Circadian entrainment

The mammalian eye contains the classical rod and cone photoreceptors necessary for the formation of images, in other words, for our sense of vision. However, the eye is also crucial for regulation of light-dependent behavioral and physiological processes that are not related to vision (Legates, 2014). Thus, the eyes perform both visual and "non-visual" functions. One essential "non-visual" function of light is synchronizing the circadian clock to daily light-dark cycles, thus creating an endogenous sense of time, and allowing physiological and behavioral processes to occur on a daily basis (Legates, 2014). While other daily cues also contribute, the strongest environmental cue in synchronizing daily (circadian) rhythms is the 24-hour light dark (LD) cycle (Golombek & Rosenstein, 2010). Non-visual photic cues maintain synchronization of circadian rhythms mainly by inducing daily phase-shifts of the underlying endogenous circadian clock, either to an earlier or later time; these shifts are referred to as phase-advances or phase-delays, respectively. These phase-shifts correct the difference between the 24-hour environmental periodicity and the circa-24-hour biological periodicity, a process which is often referred to as circadian entrainment (Pittendrigh, 1981; Golombek & Rosenstein, 2010). If the entraining period is too long or too short, ultimately exceeding the range of entrainment, the organism starts to free-run. Free running generally occurs when organisms are kept under conditions shielded from the environmental cues. This leads to circadian rhythms that no longer equal 24 hours.

The phase response curve (PRC) describes the relationship between the timing of light exposure and shifts in circadian rhythms. Studies on varying animal models have suggested that PRC is characterized by phase advance zone (around dawn), a phase delay zone (around dusk), and a "dead zone" within which the clock is insensitive to photic cues (around mid-day) (Pittendrigh, 1976; Golombek & Rosenstein, 2010). As mentioned, these phase shifts are responsible for correcting that difference between the exact 24-hour periodicity of the external environment and the approximate 24-hour periodicity of the endogenous clock. By integrating the phase-shifting effects of light across the PRC can contribute to understanding free running periods.

1.2 Masking

In addition to entrainment of the circadian pacemaker, light and darkness also have "masking" effects on behavioral and physiological processes (Mrosovsky, 1999). Thus, superimposed on any circadian influence, nocturnal animals are less active and alert when exposed to light while day-active species are less active and alert when exposed to darkness. Positive masking is defined as increased active behavior while negative masking is defined as decreased behavior. Together, entrainment and masking shape the daily profile of a circadian rhythm as expressed in the presence of a 24-hour LD cycle.

1.3 Light-dark cycles modulating mood related behaviors

Beyond their roles in circadian entrainment and masking, recent research shows that variations in daily light-dark cycles also modulate mood related behaviors. For example, several studies have exposed animals to long and short photoperiods, thus

simulating longer summer days and shorter winter days, respectively. In both nocturnal and diurnal rodents, short photoperiods generally increase anxiety- and depression- like behaviors, while long photoperiods tend to decrease anxiety- and depression- like behaviors (*Nocturnal:* Prendergast and Nelson, 2005; Pyter and Nelson, 2006; Workman et al., 2011; Workman and Nelson, 2011; Walton et al., 2012; Otsuka et al., 2014. *Diurnal:* Einat et al., 2006; Ashenazy-Frolinger et al., 2010). In addition, other studies have indicated that exposure to nocturnal illumination alleviates anxiety- and depression- like behaviors during short photoperiods (Yilmaz et al., 2004). However, there are studies that contradict these findings, including some indicating that anxiety- and depression-like behavior is increased under long photoperiods, while others indicate increases in anxiety- and depression-like behavior in *both* short and long photoperiods, relative to a standard LD 12:12 cycle (Dulcis, 2013; Weil, 2007). There are certain factors that need to be considered during the analysis of these results. For example, the studies often use different strains of mice (or even different species) and use different definitions of a "short" and "long" photoperiod duration. A study using C57BL/6 mice showed a typical pattern seen in most nocturnal and diurnal rodents, that being, increased anxiety- and depression-like behavior under a short photoperiod. Alternatively, C3H/He and CD1 mice in the same study displayed increased anxiety- and depression- like behaviors under long photoperiods (Becker et al., 2010; Flaisher-Grinberg et al., 2011).

While circadian entrainment is seen under light-dark cycles within a wide range of photoperiod durations (i.e., from seconds to about 22 hours), in the absence of a daily LD cycle (i.e., in constant darkness (DD) or constant light (LL)) circadian rhythms will free-run with non-24-h periodicity. In nocturnal animals such as mice, free-running periods

typically lengthen as a function of light intensity such that a longer than 24-hour free running period is typical under LL while a shorter than 24-hour free running period is typical under DD. Somewhat surprisingly, increases in anxiety- and depression-like behavior have been observed under both LL and DD photoperiods in rats and mice, even though LL and DD trigger opposite changes in seasonal functions (Gonzalez and Aston-Jones, 2008; Işman, Toyran & Gundogan, 2010; Tapia-Osorio et al., 2013). The fact that anxiety- and depression- like behaviors were seen in both DD and LL may suggest partially distinct mechanisms from those involved with seasonal responses to long and short photoperiods.

Additionally, studies have been done using dim light rather than complete darkness during the dark period of an LD cycle (dLAN). This was done to simulate light pollution seen in well-lit areas like cities (Bedrosian and Nelson, 2013; 2017). Unlike DD or LL lighting regimens, circadian entrainment was maintained under these conditions and did not express a free-running period. However, results show an increase in depression like behavior under a bright:dim lighting cycle in both nocturnal Siberian hamsters and diurnal Nile grass rats (Fonken et al., 2012). An ultradian LD cycle study, meaning less than 24-hour periods, has also been tested, using alternating 3.5- hour periods of light and darkness in order to examine mood-related and circadian behaviors. Under these conditions, depression-like behaviors increased despite animals having normal sleep patterns (Legates, 2012).

1.4 Light-dark cycles modulating cognitive related behaviors

Along with mood-related behaviors, shifts in the LD cycle have been shown to alter performance on cognitive-related behaviors. Studies have indicated that animals

maintained under short photoperiods display impairments in spatial learning compared to those maintained under LD 12:12 or long photoperiod conditions (Sherry et al., 1992; Perrot-Sinal et al., 1998; Healy et al., 2005; Pyter, Reader, and Nelson, 2005). It is also indicated that mice and rats maintained under constant light show impairments in the ability to locate a submerged platform in the Morris water maze task (Fujioka et al., 2011). Returning to the dLAN study, animals exposed to this lighting regimen show deficits in the Barnes maze, a popular test of spatial cognition (Fonken, Kitsmiller, Smale, & Nelson, 2012). Mice housed under ultradian LD cycles also showed deficits in spatial memory tasks, while displaying normal circadian activity and sleep patterns. Additional studies indicate disrupted spatial cognition in hamsters when exposed to a single or repeated LD phase shifts (Gibson et al., 2010). Finally, studies have shown that shifts in the LD cycle, such as those that occur in shift-workers or under jet-lag, can disrupt circadian entrainment (Elliott, 1976; Gorman et al., 2001), and alter mood- and cognition-related behaviors (Fonken, Kitsmiller, Smale, & Nelson, 2012)).

1.5 Light dark cycles modulating voluntary ethanol consumption

The effects of alterations in the daily lighting regimen and possible disruption in circadian entrainment on levels of voluntary ethanol intake has recently become an area of intense scientific interest, and is not well understood. It is been shown that human alcohol consumption varies by season and by latitude. Comorbidity is often observed between alcohol abuse and seasonal affective disorder, suggesting that photoperiods may influence human alcohol drinking. Decreased daylight during winter is associated with a higher incidence of depression and alcohol consumption, while increased daylight during summer

is associated with a lower incidence of depression and alcohol consumption (Sher, 2002; 2004; Morales-Muñoz, Koskinen & Partonen, 2017). Previous work in our lab examined voluntary ethanol intake in mice housed in either short or long photoperiods, LD 6:18 and LD 18:6, respectively (Rosenwasser, 2015). Mice displayed significantly lower ethanol intake under long photoperiods as observed in other studies (Goodwin et al., 1999; Trujillo et al., 2011; Rosenwasser and Fixaris, 2013).

Our lab most recently examined circadian activity rhythms and voluntary ethanol consumption under standard LD conditions, and under LL and DD conditions. The experiment was performed using inbred C57BL/6 and C3H/He male mice and in male and female mice of a genetically heterogeneous strain (WSC). It was discovered that ethanol intake and ethanol preference were reduced under both DD and LL in all breeding lines and both sexes (Rosenwasser and Fixaris, 2013). Due to the similarity of the effects in DD and LL, it is likely that neither circadian disruption nor a classical seasonal photoperiodic mechanism can account for these findings.

1.6 Neural pathways involved in circadian entrainment

Neuroanatomic studies have identified the photoreceptors, photopigments, and anatomical pathways involved in photic circadian entrainment (Mrosovsky 1999; Rosenwasser and Turek, 2016). Rods and cones are the classic photoreceptors and have long been thought to fullyunderlie retinal photoreception, including both the visual and non-visual effects of light. This assumption was first challenged by studies showing that circadian photoreception could occur in mice carrying a genetic mutation leading to profound degeneration of retinal rods and cones, and later, in genetically engineered mice

with a total loss of rods and cones (Provencio et al., 1998). These findings led to the recent discovery of a novel photopigment, melanopsin, expressed exclusively in a small subset of intrinsically photosensitive retinal ganglion cells (ipRGCs). Melanopsin-expressing ipRGCs have now been found to play significant roles in the effects of non-visual photic cues on both circadian entrainment and mood-related behavior (Schmidt, Chen, and Hattar, 2011; LeGates et al., 2014).

ipRGCs detect and transmit photic stimuli to the suprachiasmatic nucleus (SCN), the central "pacemaker" underlying the generation and entrainment of circadian rhythms, thereby mediating circadian entrainment. Retinal innervation of the SCN is conveyed via the retinohypothalamic tract (RHT), which is the major pathway mediating photic effects on the SCN, mainly though the release of glutamate, an excitatory neurotransmitter (Golombek & Rosenstein, 2010; Rosenwasser and Turek, 2016). Remarkably, melanopsin-expressing ipRGCs are able to maintain circadian entrainment even in the complete absence of the classical photoreceptors, rods and cones, thus showing photosensitivity when isolated from all other known retinal photoreceptors.

ipRGCs are not the only source of photic signaling directed to the SCN. The SCN also receives projections through the geniculo-hypothalamic tract (GHT) which originates from neurons of the intergeniculate leaflet (IGL) and ventral lateral geniculate nucleus (vLGN) of the thalamus. Studies have shown that GHT neurons potentially provide information regarding environmental light intensity to the SCN (Harrington), and more recently, it has been shown that the IGL and vLGN also receive retinal projections originating from ipRGCs (Fernandez, Chang, Hattar, and Chen, 2015). The SCN also receives information via the serotonergic raphe nuclei, which provide the circadian clock

with information about behavioral state, such as arousal and sleep. Studies have shown that disruption in these serotonergic fibers affects activity onset, suggesting that the raphe nuclei projections to the SCN is likely responsible for modulating timing of activity onset (Reghunandanan, 2006).

With respect to circadian entrainment, it is known that while melanopsin knockout mice ($Opn4^{-/-}$) display reduced circadian photosensitivity, these animals do continue to entrain to LD cycles, but with reduced photosensitivity, while mice with developmental loss of ipRGCs ($Opn4^{aDTA/aDTA}$) display a complete loss of circadian photosensitivity and entrainment (Altimus et al., 2010). Remarkably, these mice continue to display perfectly normal visual perception, mediated via the classical rod and cone photoreceptors. Studies have also shown circadian entrainment capacity is completely lost in absence of both the classical photoreceptors and melanopsin (Lucas et al., 2012). These observations suggest that the photopigment melanopsin contributes to, but is not necessarily needed for, circadian entrainment, while the ipRGCs are essential for entrainment. Thus, it is suggested that both the classical photoreceptors and melanopsin-expressing ipRGCs contribute to circadian entrainment in mammals and that the contribution of rods and cones to circadian entrainment appears to be mediated through synaptic contacts from rods and cones to the ipRGCs, as well. This is how the ipRGCs are able to relay signals regarding entrainment to the brain in absence of melanopsin.

ipRGC types M1-M5 are the five different types of ipRGCs that possess different structures and electrophysiological properties. Each of the five respond to photic stimulation differently. The ipRGC type that is primarily responsible for circadian photoentrainment appears to be M1. M1 is shown to provide most of the axons innervating

the SCN and the IGL. M1 is not solely responsible as it is shown that M2 projections also innervate other regions of the brain involved with non-visual light effects. Recent studies show that M1 and M2 also innervate structures dealing with mood-related behavior, like the medial amygdala and the lateral habenula. M1 and M2 also innervate structures dealing with sleep, such as the subparaventricular zone, the ventrolateral preoptic area, and the lateral hypothalamus. In addition, the SCN also innervates several major regions of the brain associated with mood-related behavior, like the medial amygdala, hippocampus, and lateral hypothalamus whereby providing a second pathway for the photic and circadian regulation of affective behavior (LeGates et al., 2014). Additional structures the SCN innervates include the ventral tegmental area, the raphe nuclei, and the septum. Each of these structures project to the hippocampus.

As discussed above, ipRGCs are responsible, directly or indirectly, for providing light information to the various brain regions involved in circadian rhythms, sleep, mood, and cognition. However, while the evidence linking melanopsin and ipRGCs to circadian entrainment is strong, the evidence for the role of melanopsin signaling in the photic regulation of mood- or cognitive-related behavior is much more preliminary. As described above, our laboratory has recently shown that voluntary alcohol (ethanol) intake is suppressed under either constant light (LL) or constant darkness (DD) relative to mice held under a standard entraining light-dark (LD) cycle. Because this effect is likely to be mediated by both the circadian pacemaker and by anxiety- and depression-like mood states, we designed the present experiments to examine the role of melanopsin signaling and ipRGCs in mediating the effects of environmental lighting on voluntary ethanol intake, using melanopsin knockout ($Opn^{CRE/CRE}$) mice developed and provided to us by

collaborators at Johns Hopkins University. Studying the role of melanopsin signaling in the photic regulation of alcohol intake may contribute to the overall understanding of the effects of season, latitude, and the circadian clock on mood-related human behaviors, such as alcoholism and seasonal affect disorder (SAD) (Pittendrigh, 1976; Golombek & Rosenstein, 2010). We hypothesize that melanopsin deletion ("knockout") will attenuate the effects of environmental lighting on voluntary ethanol intake, similar to their known effects on circadian entrainment. To test this hypothesis, we will pursue the following Specific Aims:

1. To confirm previous findings from our laboratory showing that voluntary alcohol (ethanol) intake is suppressed in mice housed under either constant light (LL) or constant darkness (DD), relative to animals standard light-dark (LD) cycles.
2. To examine the role of melanopsin signaling in mediating the effects of environmental lighting on voluntary ethanol intake, using a genetic model developed at Johns Hopkins University in which the gene for melanopsin synthesis has been deleted (*Opn4*$^{Cre/Cre}$).

EXPERIMENTAL PLAN

2.1 Overview

The experiment conducted compared *Opn4*$^{CRE/CRE}$ to wild-type controls of the same genetic background (B6x129) under varying lighting conditions to collect data on circadian activity rhythms and voluntary ethanol intake.

2.2 Genetic models

As mentioned above, ipRGCs not only transmit non visual photic information utilizing the photopigment melanopsin, but also relay signals from the classical photoreceptors to certain brain regions. To account for the two distinct processes at hand, the Hattar lab (originally at Johns Hopkins, now at the NIH) generated two lines of mutant mice. In one of these models, melanopsin signaling is blocked by genetic deletion of the melanopsin gene (Opn4), and in the other model, a diptheria toxin-coupled transgene is used to developmentally ablate the ipRGCs. In this second model, not only is melanopsin signaling prevented, but the relay of signals from rods and cones to the SCN via ipRGCs is also blocked.

Melanopsin is the protein product of the *Opn4* gene. Mice unable to produce melanopsin were generated by replacing part of the *Opn4* gene with a "knock-in" that prevents *Opn4* transcription. In this case, CRE replaces part of the coding region of the *Opn4* gene, resulting functionally in a melanopsin knockout (*Opn4*$^{Cre/Cre}$/*Opn4*$^{-/-}$). The *Opn4* gene is located on the 14th chromosome, 34590618-34600142 bp. CRE recombinase is inserted immediately after the start codon of the *Opn4* gene, replacing the open reading

frame. Homologous recombination in ES cells results in replacement of the coding region of *Opn4* with the CRE construct. This is the model that was employed in this experiment.

2.3 Mating and weaning pups

Experimental animals were bred in-house from mated pairs supplied by Dr. Samer Hattar, Johns Hopkins University. Mice were placed in individual cages for acclimation to new living conditions. There were four mating pairs (female N=4, and male N=4) to produce progeny for experimental testing. After birth all pups remained with the mother mouse for twenty-one days, and then weaned. During the weaning process, all progeny were placed in standard mouse cages without running wheels until they were eight weeks old. Female progeny in the same litter were housed together while male progeny were housed individually. Once the mice reached maturity, they were housed individually in running wheel cages.

2.4 Housing experimental animals

Experimental animals were placed in standard mouse cages with functioning running wheels. All running wheel cages were placed in sound attenuating cabinets equipped with computer-controlled light fixtures. Lighting regimens were controlled using ClockLab computer interface system. Food, ethanol, and water were made freely available for the entirety of the experiment. Ethanol and water bottle positions were alternated daily for the duration of the experiment, in efforts to minimize possible side preferences.

2.5 Acclimation to running wheels

Female and male control (B6/129) and *Opn4*$^{CRE/CRE}$ mice were individually assigned to running wheel cages located in either housing cabinet 1 or 2. Mice were assigned to running wheel cages by alternating sex and genotype until each cabinet was full. Thus, each cabinet contained mice from each of four distinct groups: *female KO, female WT, male KO, and male WT*. Cabinet 1 housed the "DD group" while cabinet 2 housed the "LL group", as described below. The body weight of each mouse was measured before being placed in their running wheel cage and thereafter on a weekly interval. Daily checks on all animals were performed to check health, wellness, and living conditions.

2.6 Lighting regimens

All mice were initially maintained under the same LD 12:12 schedule for three weeks (LD1), followed by three weeks in either DD or LL in separate groups, and then returned to LD2 for a final three weeks.

2.7 Measurements

ClockLab hardware and software was used to monitor and analyze running-wheel activity, Activity patterns were visualized in "actogram" plots, and activity measures included the free-running period and the number of daily wheel turns for each animal under each lighting condition. Ethanol and water measurements were made by recording the weight of all bottles after filling them with 10% ethanol solution or water and then weighing the bottles again at weekly intervals. Ethanol intake was determined by grams of ethanol per kg body weight per day, based on one gram of 10% ethanol solution containing .079 grams

of ethanol. Water intake was measured as milliliters of water consumed per day. Ethanol preference was determined by dividing the weight of ethanol solution consumed by the total weight of fluid (ethanol + water) consumed at the end of each week and was reported as a percentage of the total amount of fluid consumed.

2.8 Statistical Analysis

Data was analyzed using a factorial mixed design ANOVA, with lighting condition (LD-LL-LD or LD-DD-LD) as a within-subject factor and genotype (wild-type control or melanopsin knockout) and sex (male or female) as between-group factors. Separate ANOVAs were performed for each of the two lighting groups (LL group vs DD group) because the main purpose of the experiment was to detect genotype by lighting condition interactions, but not to compare LL to DD directly.

RESULTS

3.1 Circadian period

As expected from previous studies, WT control (B6x129) and *Opn4*$^{-/-}$ male and female mice showed similar stable circadian entrainment patterns under initial LD conditions, expressing large amounts of nocturnal activity and much less activity during the light phase(Fig. 1). Also as expected, control mice displayed free-running circadian periods shorter than 24 hours under DD and longer than 24 hours under LL conditions (Fig. 2). While *Opn4*$^{-/-}$ mice and control mice displayed similar free-running periods in DD, *Opn4*$^{-/-}$ mice showed a significantly blunted period-lengthening effect and much shorter periods than control mice under LL conditions. These observations confirm that the elimination of melanopsin photoreception in *Opn4*$^{-/-}$ mice results in decreased circadian photosensitivity.

Analysis of circadian period under LL conditions:

Analysis of the circadian activity using ANOVA generally confirms the observations described above. In the LL group, lighting condition had a significant effect on free-running period ($F=40.075$, $p<.001$), meaning that photoperiods lengthened in both strains. However, a significant lighting condition by strain interaction ($F= 13.844$, $p<.001$) confirmed that LL had a less drastic lengthening effect in *Opn4 -/-* mice compared to control mice.

Analysis of circadian period under DD conditions:

In the DD group, lighting condition had a small, yet significant ($F=3.847$, $p=.027$) effect on free-running period, meaning that periods shortened under DD in both strains.

Unlike in the LL group, however, there was no lighting condition by strain effect, meaning that the effects of DD were indistinguishable in *Opn4-/-* mice and controls.

3.2 Wheel turns per day

Analysis of wheel turns per day under DD conditions:

ANOVA showed wheel turns per day to generally decrease across lighting condition (LD-DD-LD) ($F=4.035$, $p<.023$) in both strains (Fig. 3). In addition, WT control mice showed more wheel turns per day than *Opn4 -/-* mice across the lighting conditions ($p<.001$).

Analysis of wheel turns per day under LL conditions:

ANOVA analysis showed the lighting condition by sex by strain interaction to be significant ($p<.047$), meaning that male control and *Opn4 -/-* mice differed over lighting conditions while female mice did not (Fig. 3).

3.3 Ethanol intake

Analysis of ethanol intake under DD conditions:

In general, ethanol intake was decreased under both DD and LL conditions, relative to LD 12:12, in all sexes and strains. In the DD group, ANOVA showed ethanol intake varied significantly across lighting conditions (LD-DD-LD) ($F=27.229$, $p<.001$) in both strains, while the lighting condition by strain interaction was non-significant, meaning that reductions in ethanol intake under DD were similar in both control B6/129 and *Opn4 -/-* mice (Fig. 4). Finally, as expected from numerous previous studies, females consumed

significantly more ethanol than male mice (F=34.382, p<.001) across all lighting conditions.

Analysis of ethanol intake under LL conditions:

As in DD, ANOVA showed that ethanol intake significantly varied over lighting condition (LD-LL-LD) in the LL group (F=24.648, p<.001) in both strains, while a non-significant lighting condition by strain interaction showed that ethanol intake was reduced similarly under LL conditions in both control B6/129 and *Opn4 -/-* mice (Fig. 4). Also similar to the DD group, females in the LL group consumed significantly more ethanol than male mice (F=107.203, p<.001).

3.4 Ethanol preference

Analysis of ethanol preference under DD conditions:

In general, ethanol preference gradually increased across lighting conditions (LD-DD-LD), with the exception of WT control males (expressing significant decrease under DD compared to LD1 and LD2). ANOVA analysis showed ethanol preference varied significantly across lighting condition (F=16.531, p<.000) in both strains (Fig. 5). Additionally, lighting condition by strain interaction was significant (F=.3.677, p<.030), meaning that *Opn4*$^{-/-}$ mice show gradual increase increase over lighting condition while WT control mice showed lowest ethanol preference during DD. Females expressed a greater preference compared to males (F=28.571, p<.000). Lastly, ANOVA analysis showed a significant sex by strain interaction (F=17.204, p<.000), meaning female control mice had a greater preference compared to *Opn4*$^{-/-}$ mice, and male *Opn4*$^{-/-}$ mice had a greater preference than control mice.

Analysis of ethanol preference under LL conditions:

Both strains of female mice showed a gradual increase in ethanol preference, while both strains of male mice showed decrease in ethanol preference. In the LL group, ANOVA analysis showed ethanol preference to be significantly varied across lighting condition (LD-LL-LD) ($F=18.103$, $p<.000$) in both strains, while the lighting condition by strain interaction was non-significant ($F=.344$, $p<.710$), meaning that the trend of ethanol preference under LL were similar in both B6/129 and *Opn4 -/-* mice (Fig. 5). Lastly, lighting condition by sex interaction was significant ($F=5.488$, $p<.006$), indicating the gradual change over lighting condition in females compared to the decrease in ethanol preference in males.

3.5 Water intake

Analysis of water intake under DD conditions:

In general, water intake was decreased under both DD and LL conditions, in all sexes and strains. In the DD group, ANOVA showed water intake varied significantly across lighting conditions (LD-DD-LD) ($F=18.696$, $p<.000$) in both strains. In addition, lighting condition by strain interaction was significant ($F=4.050$, $p<.021$), indicating the drastic water intake reduction in *Opn4*$^{-/-}$ mice compared to WT control mice across lighting condition (Fig. 6). ANOVA also showed that males consume more water than females ($F=26.605$, $p<.000$). Control mice generally drank more water than *Opn4*$^{-/-}$ mice ($F=9.762$, $p<.003$). Lastly, ANOVA showed sex by strain interaction was significant ($F=15.096$, $p<.000$), meaning both female strains has similar water consumption while male WT controls consumed more water compared to *Opn4*$^{-/-}$ mice.

Analysis of water intake under LL conditions:

Similar to DD, ANOVA analysis showed water intake was significantly varied over lighting condition (LD-LL-LD) ($F=32.779$, $p<.000$) in both strains (Fig. 6). However, lighting condition by strain interaction was not significant ($F= 2.164$, $p<.122$), indicating that water intake was reduced similarly under LL conditions in both strains. As in DD, males generally drink more water than females ($F=52.187$, $p<.000$).

DISCUSSION

4.1 Interpretations of results

As expected from previous studies (Altimus et al., 2010), melanopsin knockout (*Opn4*$^{-/-}$) mice in the present study showed reduced circadian photosensitivity relative to control (B6x129) mice, as evidenced by the significantly blunted period-lengthening seen in knockouts under LL. Also as seen in previous studies, melanopsin knockout and control mice showed very similar circadian activity patterns in both LD and DD, indicating that the loss of melanopsin alters circadian photosensitivity but not basic circadian pacemaker function. These observations confirm at the behavioral level that the mice used in the present experiment were indeed melanopsin-deficient.

The present results also confirm previous reports from our laboratory that voluntary ethanol intake in mice is reduced in both DD and LL, relative to standard LD 12:12 conditions, and extend those results to previously untested genotypes. Most importantly, the lack of significant strain by lighting condition interactions in either the LL or DD group in the present study indicates that melanopsin knockout and control mice showed essentially identical reduction in ethanol intake under both DD and LL. While these results suggest that melanopsin-dependent photoreception does not contribute to the effects of lighting conditions on voluntary ethanol intake, it is also possible that rod- and cone-dependent photoreception is able to fully compensate for the loss of melanopsin in *Opn4*$^{-/-}$ mice.

Ethanol preference varied from ethanol intake data. It might seem counterintuitive that ethanol preference and intake did not correspond; however, this trend is a result of decreased water intake. Careful examination of the data shows that while some groups

(mainly the males) showed reductions in ethanol preference under LL and/or DD, other groups (mainly the females) showed progressive increases in preference over the course of the experiment. These increases were accompanied by progressive and unexpected decreases in water intake over the experiment, which partially confounded the assessment of ethanol preference. Water intake can unintentionally effect ethanol preference, as preference was determined by dividing the weight of ethanol solution consumed by the total weight of fluid (ethanol + water) consumed at the end of each week.

4.2 Limitations of running wheel cages

Something to consider is that the animals were kept in running-wheel cages for the duration of the experiment. While this allows us to effectively collect data on circadian entrainment patterns under the varying lighting regimens, previous studies show that running wheel activity can alter voluntary ethanol intake in mice. ((McMillan et al., 1995; Ehringer et al., 2009; Piza-Palma et al., 2014) In our lab, findings show that mice kept in running wheel cages typically show an increase in water intake, leading to a decrease in ethanol preference, but no significant increase or decrease on voluntary ethanol intake (Rosenwasser et al., 2012; 2015). Another previous study done in Ehringer's lab found that the reduction in ethanol preference in mice kept in running wheel cages is a result of increased water intake and not due to changes in ethanol consumption. However, Ehringer's lab also have done studies that do show reduction in ethanol intake as a result of running wheel cages. In this study, changes in ethanol preference seemed to be accompanied by changes in water intake rather than ethanol

intake. Thus, this information suggests that decreased voluntary ethanol intake in mice held in running wheel cages is not a result of the wheels themselves.

4.3 Limitations of water bottles

In this study, standard glass water bottles with rubber stopper and sipper tubes were used. This is, inevitably, going to produce error as liquid, both ethanol and water, leak out of the sipper tubes when moved. Inevitably, error is formed due to this in some extent. To make this experiment more precise, additional equipment would be necessary to dispense the fluid to eliminate error by fluid loss.

4.4 Decrease in ethanol intake indicating anxiety- and depression-like behavior

As mentioned, numerous studies have been performed in efforts to identify the effects of varying lighting regimen on cognitive- and/or mood-related behavior; including studies of the effects of the ultimate dark or ultimate light cycle, DD and LL, respectively. Many studies suggest that housing under atypical light-dark cycles can result in increased anxiety- and/or depression-like behaviors, relative to animals housed in standard LD 12:12 conditions, in a wide variety of animal models (Stephenson et al., 2012; Landgraf et al., 2014; LeGates et al., 2014). In this study, varying level of voluntary ethanol intake was used in order to indicate anxiety- and depression-like behavior. Anxiety- and depression-like behavior indicated by reduction in voluntary ethanol intake may seem counterintuitive as it might be expected that increased voluntary ethanol intake would be observed.

The complexity of the relationship between stress and alcohol consumption is not well understood. Motivation for increased alcohol consumption may occur in efforts to

alleviate stress as it is an effective anxiolytic. However, alcohol is known to activate the hypothalamic- pituitary-adrenocortical (HPA) axis which plays a major role in stress response. Several factors can influence the magnitude of the response induced by alcohol, including, genotype, gender, and host. Varying studies have been performed that indicate increased, decreased, and little-to-no change in voluntary ethanol consumption as a result of stressors (Becker et al., 2011).

The mechanisms contributing to stress-induced decreases in ethanol intake are not well understood. Studies have been done using rats possessing a higher ethanol preference due to a variety of factors showing a greater likelihood of expressing stress induced reduction in ethanol intake. Factors include altering the taste and sweetness of ethanol, forced consumption, varying natural differences in intake preference, and genotype differences for high ethanol intake (Becker et al., 2011).

Chronic stress exposure has varying results based off strain, animal model, and stressor type. Very few studies testing voluntary ethanol intake level changes due to shifts in circadian cycles have been done. Adult male Sprague-Dawley rates showed significant increase in voluntary ethanol intake when exposed to a single 8 hour shift in the LD schedule (Becker et al., 2011). Repeated changes in light schedule also showed an increase in voluntary ethanol intake. However, male and female Fisher and Lewis rats showed a significant decrease in voluntary ethanol intake when exposed to 6 hour shifts in the LD cycle. Male and female HAD1 rats exhibit a similar trend as Fisher and Lewis rats. Additionally, a study using C57BL/6J and HAP2 mice (genetically possessing a high ethanol preference), and LAP2 mice (genetically possessing a low ethanol intake) all showed reduction in voluntary ethanol intake when exposed to shifts in their lighting

regimen. Most prevalent to the conditions in this study, male Wistar rats held in DD or LL showed significant reductions in voluntary ethanol intake as well (Becker et al., 2011). Thus, even though the underlying mechanisms are not understood, many studies indicate that stressors mainly lead to reduction in voluntary ethanol intake, especially in studies using free choice drinking in nondependent animal models.

4.5 B6x129 mice

To account for the variations in ethanol consumption mentioned above, this study used control mice with the same genetic background as the melanopsin knockout mice . This avoids varying ethanol preferences between strains. B6x129 mice display a genetically high ethanol intake compared to other strains. Thus indicating an acceptable strain to use as control mice in this experiment and suggesting that notable decrease in voluntary ethanol consumption in these mice is likely an anxiety-like behavior due to being held under stressful lighting conditions. However, because the mutant and wild-type mice were not littermates, the wild-type mice employed here must be considered to be "approximate controls". The mice used in this experiment were F1 hybrids, meaning they are heterozygous at all loci. Ideally, F2 hybrid controls would have been used. F2 hybrids represent a unique recombination of the two parental genomes; F2 can either be B6/B6, B6/129, or 129/129 at any locus. This makes F2s generally preferred because they represent genetic diversity in the mutant line to a better extent.

4.6 Role of melanopsin signaling

The mechanisms underlying the consistent reduction in voluntary ethanol intake in both LL and DD are unclear. The effects of LL and DD on circadian disruption are drastically different. The effects of LL on circadian disruption tend to lengthen the 24 hour period more drastically than the shortening of the 24 hour period in DD animals. Indicating, it seems unlikely that circadian disruption accounts for the significant decrease in voluntary ethanol intake in both LL and DD. Thus, this current study looked at melanopsin signaling. Studies have shown that melanopsin is not essential for the SCN, or the circadian clock, to receive photic information as rods and cones relay photic information to the SCN via retinal ganglion cells. However, melanopsin signaling contributes significantly to the magnitude of photic responses. Melanopsin mice exhibit decreased circadian photosensitivity. In DD, melanopsin knockout and wild type mice have roughly the same free running period length around 23.8 hours. In LL, melanopsin knockout mice show a decreased free running period compared to wild type mice, 24.3 and 25 hours, respectively (Fig. 2).

However, the results collected showed that voluntary ethanol intake is reduced in both DD and LL, relative to standard LD conditions, in melanopsin knockout mice as well as control mice. The data did not show the expected "blunted" effect in the melanopsin knockout mice. Meaning, melanopsin signaling did not play a significant role in mediating the effects of lighting regimen on voluntary ethanol intake. The similar reduction in voluntary ethanol intake in DD and LL are hard to account for by a single underlying mechanism as many factors may contribute to this phenomenon.

4.7 Further studies

Currently the Rosenwasser lab is performing a study involving mice with developmental loss of ipRGCs ($Opn^{aDTA/aDTA}$). It is known that mice with developmental loss of ipRGCs display a complete loss of circadian photosensitivity. Meaning these mice are sighted and receive visual input, but are not receiving any input regarding non-visual light cues. Therefore, they cannot entrain to a circadian rhythm. Therefore, it is hypothesized that these genetic manipulations will eliminate the effects of environmental lighting on voluntary ethanol intake, similar to their known effects on circadian entrainment.

This study will have a similar protocol used in this experiment. $Opn^{aDTA/aDTA}$ and control mice will be held in the same cabinets as the mice in this experiment. Mice will be subjected to 3 weeks of an LD cycle, 3 weeks of LL or DD, and finally, 3 weeks of a second LD cycle. Ethanol and water measurements will be collected on a weekly interval. Circadian patterns and wheel turn will be collected continuously via ClockLab.

4.8 Conclusion

Understanding the mechanisms underlying the deficits of cognitive and mood related behavior while maintained under varying lighting regimen can help further understand the effects of lighting on humans. To date, treatment for mood disorders are limited. Development of more effective treatments are required. In order to do so, connections of the ipRGCs and their influence on mood and cognitive related behaviors need to be studied further in efforts to understand the influence of light. Understanding the interaction between light and complex behavior may lead to more effective lighting

schedules in everyday lives. Performing experiments using varying wavelengths of color on mood and cognitive related behavior may contribute to better engineering of lights for brightly lit cities and lights for homes.

FIGURES

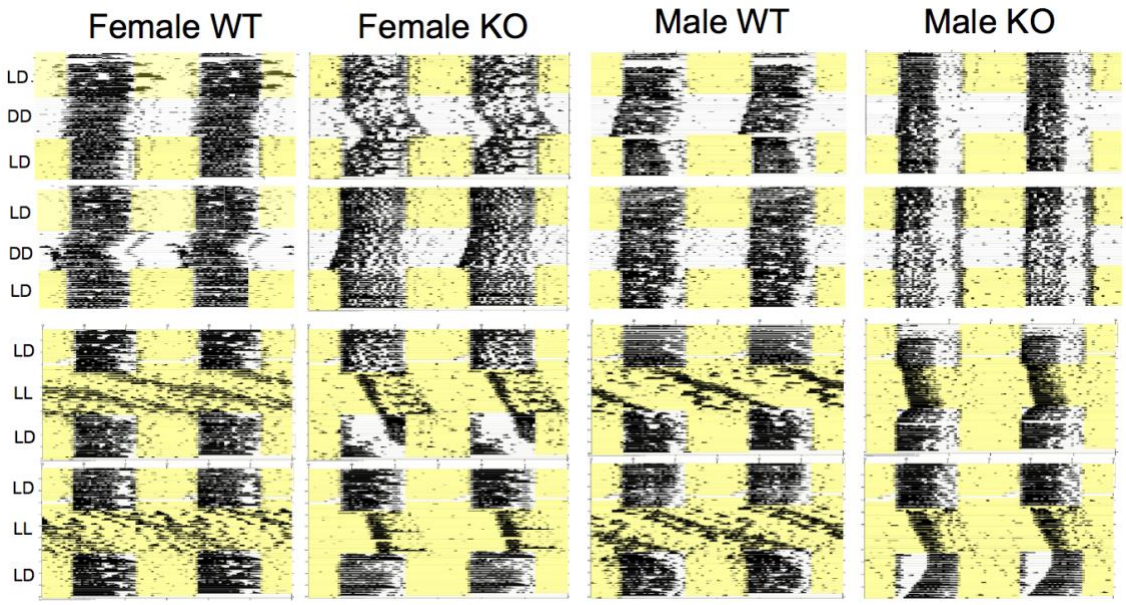

Figure 1. Double-plotted (48-hour span) circadian activity records for two representative animals from each strain (B6/129; Opn4-/-), group (DD; LL), and sex (male; female). Y-axis represents days, X-axis represents hours. Yellow regions indicate light exposure, while white areas indicate absence of light exposure. Shaded black areas indicate activity level of animal.

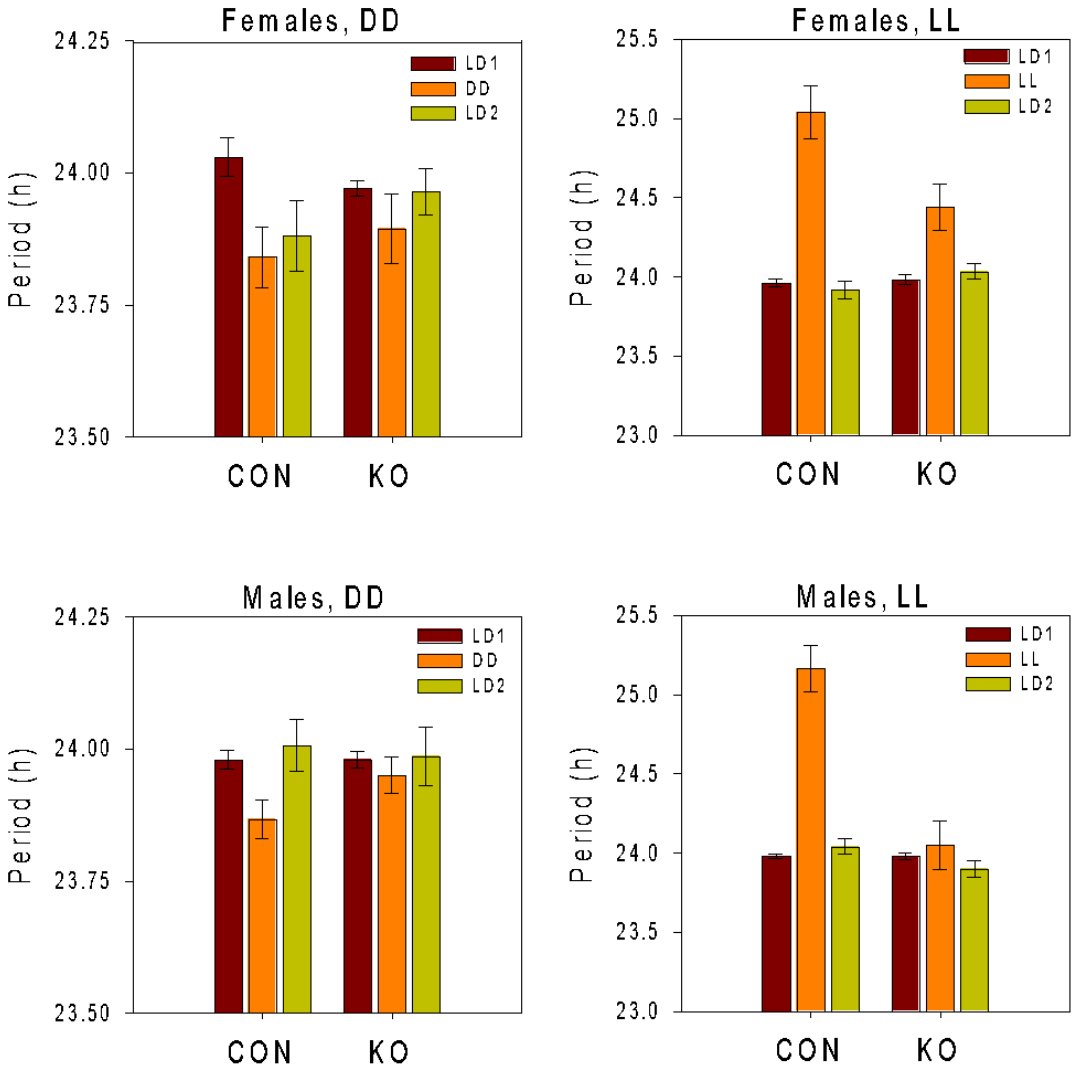

Figure 2. Entrainment period (hours) in male and female B6/129 control mice (Con) and Opn4-/- mice (KO) during exposure to the first period using a standard 12:12 light-dark cycle (LD), to either constant light (LL) or constant dark (DD), and the second period using a standard 12:12 light-dark cycle.

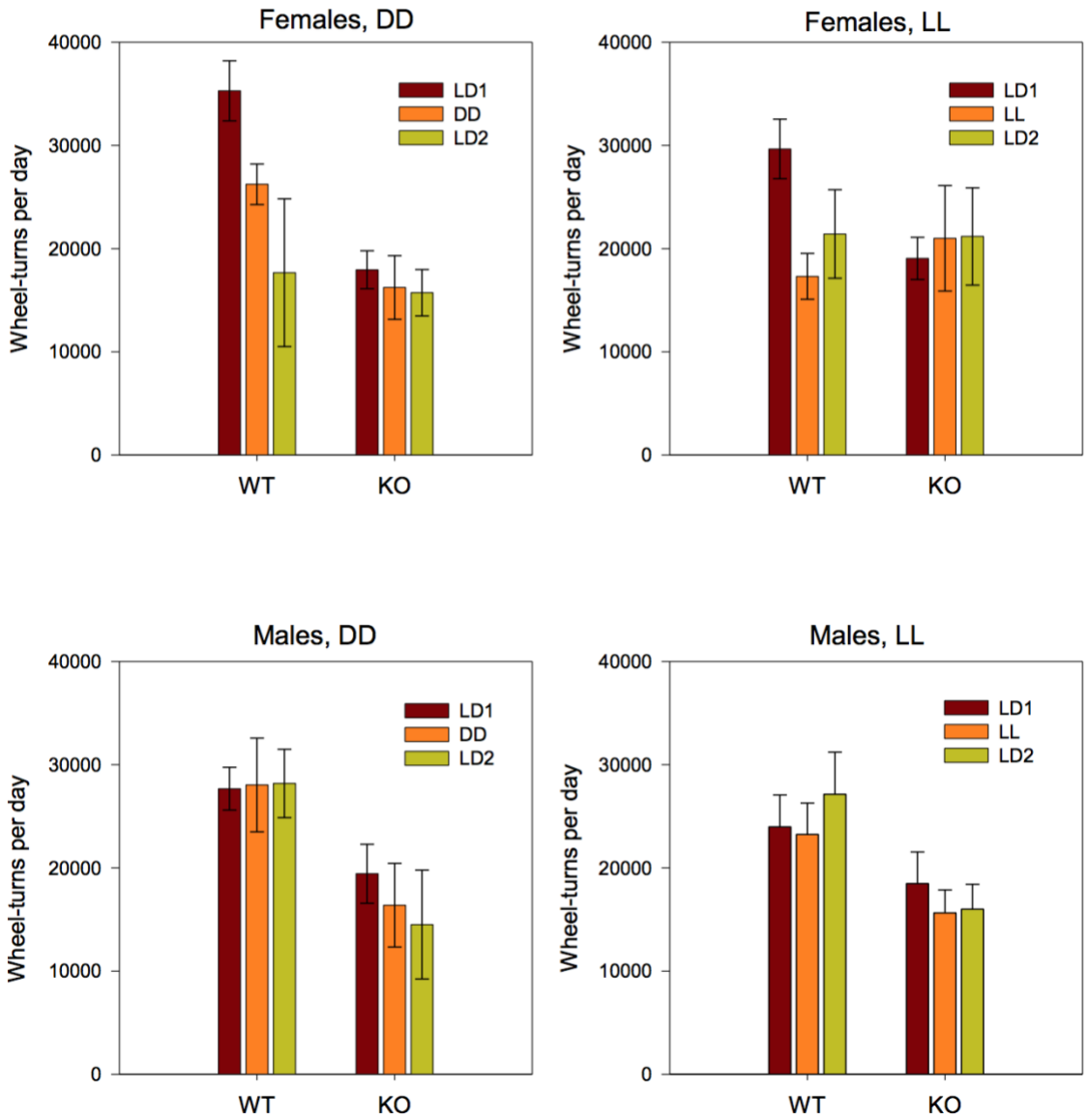

Figure 3. Wheels turn per day in male and female B6/129 control mice (WT) and Opn4-/- mice (KO) during exposure to the first period using a standard 12:12 light-dark cycle (LD), to either constant light (LL) or constant dark (DD), and the second period using a standard 12:12 light-dark cycle.

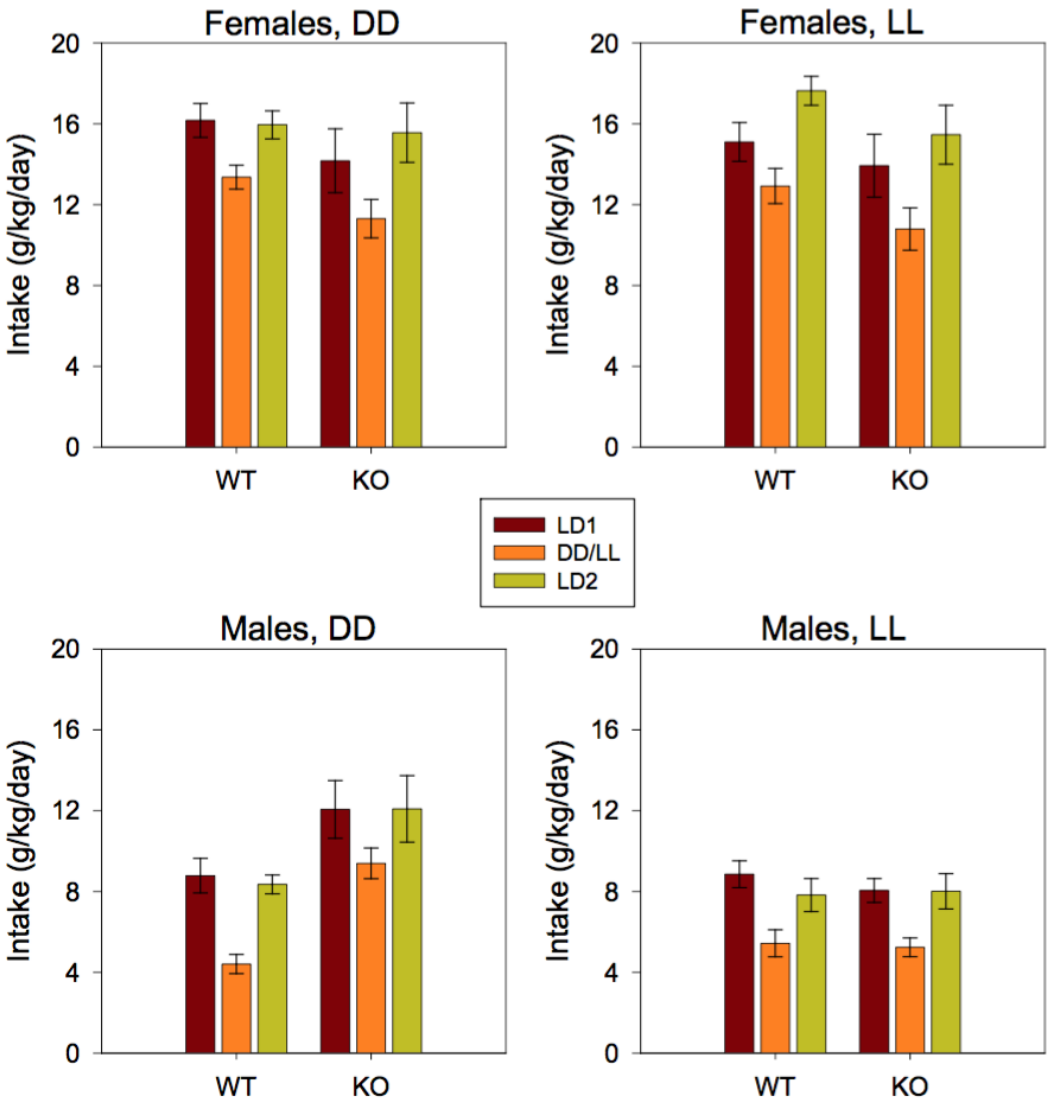

Figure 4. Ethanol intake (g/kg/day) in male and female B6/129 control mice (WT) and Opn4-/- mice (KO) during exposure to the first period using a standard 12:12 light-dark cycle (LD), to either constant light (LL) or constant dark (DD), and the second period using a standard 12:12 light-dark cycle.

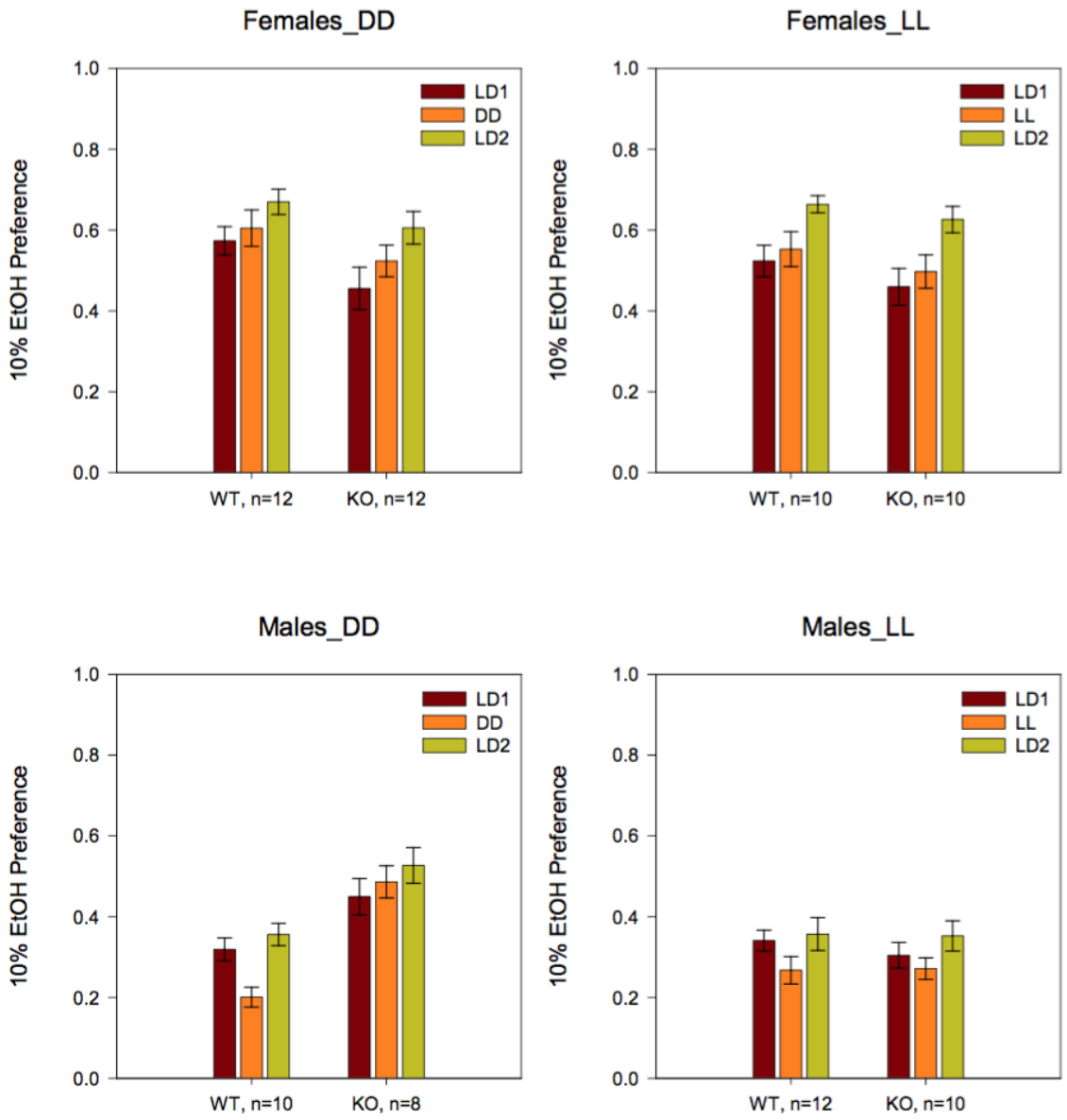

Figure 5. Ethanol preference in male and female B6/129 control mice (WT) and Opn4-/- mice (KO) during exposure to the first period using a standard 12:12 light-dark cycle (LD), to either constant light (LL) or constant dark (DD), and the second period using a standard 12:12 light-dark cycle.

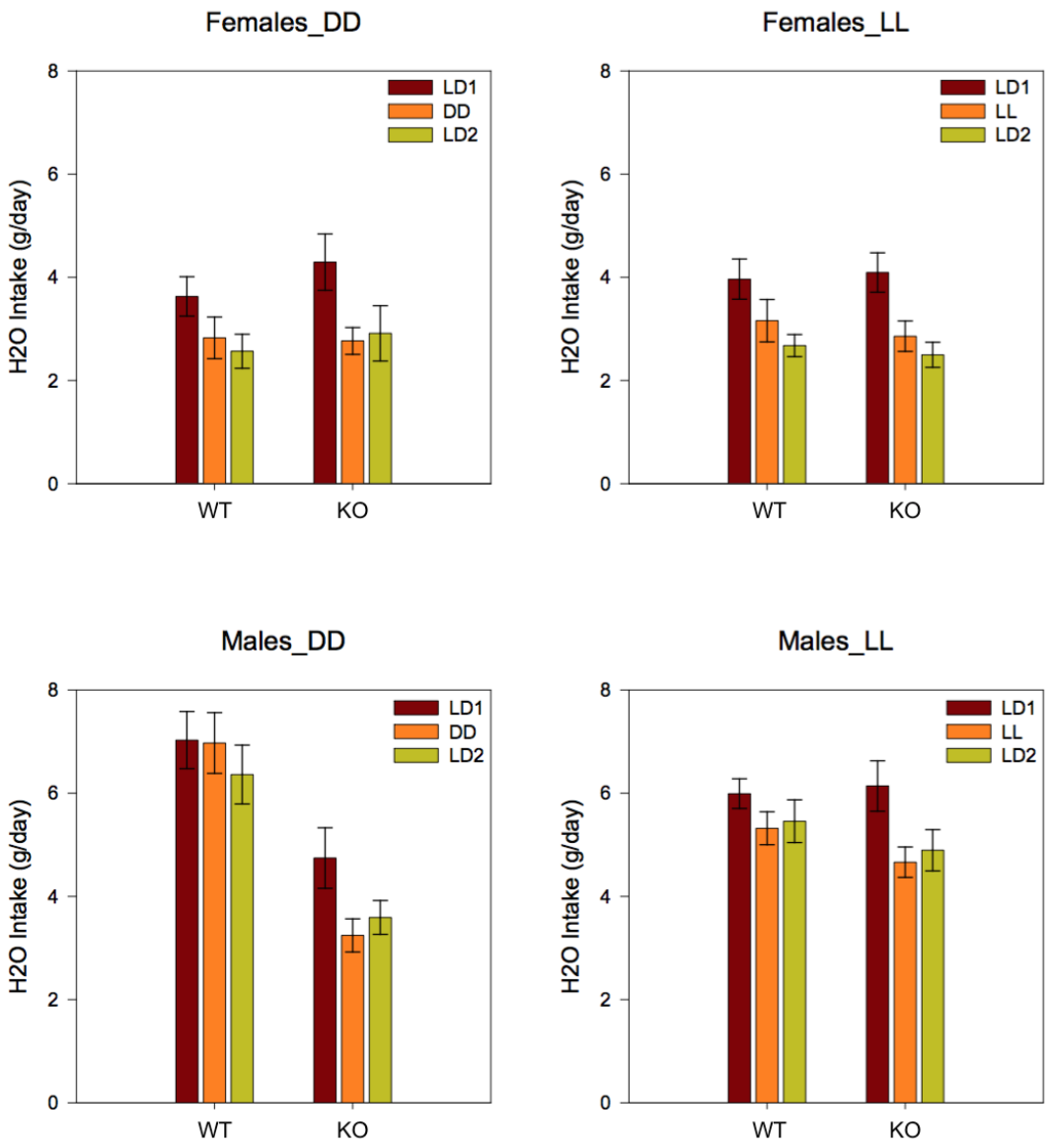

Figure 6. H2O intake (g/day) in male and female B6/129 control mice (WT) and Opn4-/- mice (KO) during exposure to the first period using a standard 12:12 light-dark cycle (LD), to either constant light (LL) or constant dark (DD), and the second period using a standard 12:12 light-dark cycle.

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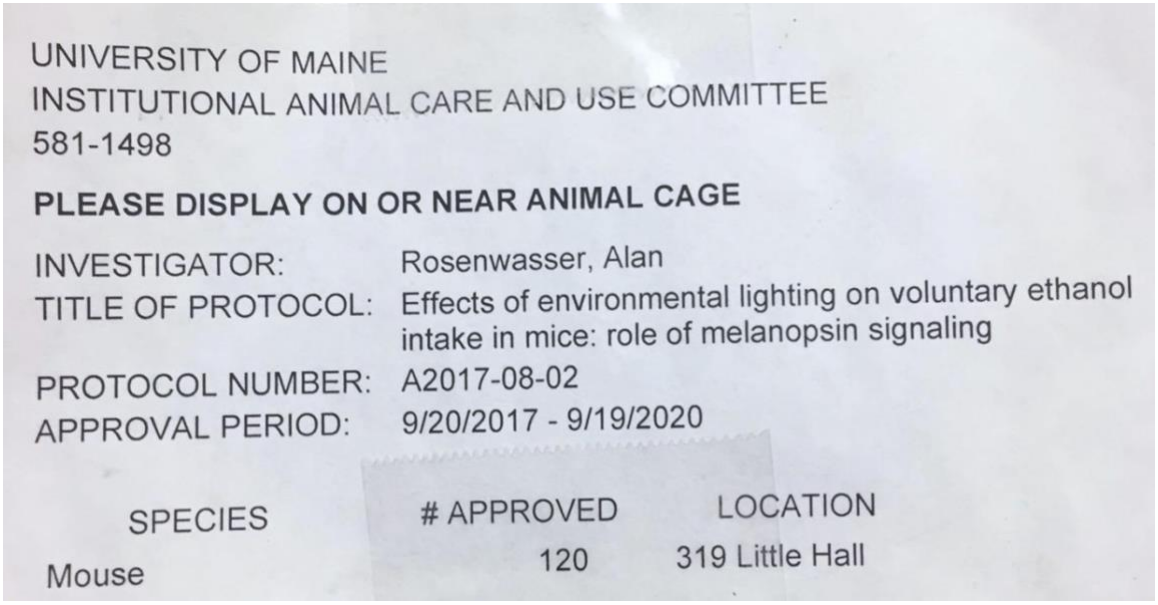
APPENDIX

APPENDIX: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) APROVAL



UNIVERSITY OF MAINE
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
581-1498

PLEASE DISPLAY ON OR NEAR ANIMAL CAGE

INVESTIGATOR:	Rosenwasser, Alan
TITLE OF PROTOCOL:	Effects of environmental lighting on voluntary ethanol intake in mice: role of melanopsin signaling
PROTOCOL NUMBER:	A2017-08-02
APPROVAL PERIOD:	9/20/2017 - 9/19/2020

SPECIES	# APPROVED	LOCATION
Mouse	120	319 Little Hall

AUTHOR'S BIOGRAPHY

Rachel M. Brooks was born in Bangor, Maine on August 12th, 1997. She was raised in Clifton, Maine and graduated from John Bapst Memorial High School in 2015. Majoring in Biology, Rachel has a minor in psychology. Rachel received an INBRE fellowship award. Upon graduation, Rachel plans to work at Acadia Hospital before returning to work on an advanced degree in clinical psychology.